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Molecular Characterization of a cDNA Encoding *Slc11A1* (*Nramp1*) of Native Thai Cattle

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ABSTRACT

Solute carrier family 11 member A1 (*Slc11a1*) is a candidate gene associated with resistance to intracellular pathogens. The complimentary DNA (cDNA) encoding the complete *Slc11a1* protein was cloned and sequenced from 10 individuals in two breeds of Thai native cattle (White Lamphun and Ko Lan). The 1773–1790 bps of cloned *Slc11a1* cDNA consisted of an open reading frame of 1,641 bps and 1,644 bps which coded for a complete protein of 547 and 548 amino acid residues. Hydrophobicity analysis of the encoded protein revealed 12 transmembrane regions. One predicted extracellular glycosylated loop, a consensus transport motif and several consensus phosphorylation sites were observed. At amino acid levels, Thai cattle *Slc11a1* exhibited 99.3–99.8% and 99.3–100% identity with that of Zebu (*Bos indicus*) and European (*Bos taurus*) cattle, respectively. Six previously reported nucleotide substitution variants and one 3-nucleotide deletion variant within the coding region were identified. Genotyping of the $(GT)_n$ microsatellite polymorphism within the 3' UTR revealed three alleles (GT_{10} –GT_{12}) in the GT block 1 and four alleles (GT_{13} –GT_{16}) in the GT block 3. The data provide additional sequence information useful for further investigations on possible correlations between allelic variants of *Slc11A1* gene and disease resistance in Thai cattle.

Keywords: native cattle, *Slc11A1*, *NRAMP1*, disease resistance, polymorphisms

INTRODUCTION

Solute carrier family 11 member 1 (*Slc11a1*) or formerly natural resistance-associated macrophage protein 1 (*Nramp1*) is an integral membrane protein of the lysosomal compartment of macrophages. After phagocytosis, the *Slc11a1* is recruited to the membrane of the microbe-containing phagosome and works as a proton/divalent cation transporter that affects resistance to intracellular pathogens and influences antigen presentation (Blackwell *et al*., 2001; Forbes and Gros, 2001). First evidence in mice showed that glycine to aspartate substitution at position 169 (p.G169D) within the fourth transmembrane domain of *Slc11a1* results in increased susceptibility to intracellular pathogens including *Leishmania donovani*, *Salmonella typhimurium* and *Mycobacterium bovis* (Vidal *et al*., 1995). Although the anti- pathogenic activity of *Slc11a1* remains unclear, numerous studies in humans and various animal species showed that allelic variants at the *Slc11a1* homologue were associated with susceptibility to infection with pathogens (Vidal *et al*., 1993; Sanchez-Robert *et al*., 2008).

In cattle, a bovine homolog of mouse *Slc11a1*, designated bovine *Slc11a1* has been

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identified on the bovine chromosome BTA 2 and the complimentary DNA (cDNA) clone had 86.9% sequence identity with mouse *Slc11a1* (Feng *et al.*, 1996). The predicted bovine *Slc11a1* protein contains 548-amino-acid residues and has 12 putative transmembrane domains (TM), two N-linked glycosylation sites and a conserved consensus transport motif located between the predicted TM domains 8 and 9. Northern blot analysis showed that bovine *Slc11a1* was expressed mainly in macrophages, the spleen and the lung (Feng *et al.*, 1996). High-level expression of *Slc11a1* protein was also observed in peripheral blood cells and granuloma cells of tuberculous cattle (Estrada-Chávez *et al.*, 2001).

Several polymorphisms have been described in the bovine *Slc11a1* gene, and the association of the variation of the *Slc11a1* gene with natural resistance or susceptibility to some specific diseases has been reported in cattle (Adams and Templeton, 1998; Horín *et al.*, 1999; Barthel *et al.*, 2001). Horín *et al.* (1999) showed a variation in the repeat number on a guanine-thymine (GT_n) dinucleotide microsatellite in the 3' untranslated region (3'UTR) of bovine *Slc11a1* gene. Based on single-strand conformation polymorphism (SSCP) analysis, the impact of the $(GT)_n$ sequence variant on natural resistant against brucellosis was demonstrated *in vivo* (Adams and Templeton, 1998; Martinez *et al.*, 2008b). *In vitro*, Barthel *et al.* (2001) showed that a polymorphism within a microsatellite in the 3'UTR is strongly associated with the expression of the bovine *Slc11a1* gene and the control replication of *Brucella abortus* in murine RAW264.7 macrophage. However, there are also contradictory results regarding the association between 3'UTR polymorphism and resistance to brucellosis in cattle (Kumar *et al.*, 2005; Paixão *et al.*, 2007). Although it is not clear how microsatellite $(GT)_n$ polymorphism at 3'UTR influences *Slc11a1* function, multiple variants within the coding and other regions of *Slc11a1* gene have been identified, with little or no attention paid to the potential of these variants for their association with disease resistance. In addition, variation of the *Slc11a1* sequence has been shown among cattle breeds (Ables *et al.*, 2002; Martinez *et al.*, 2008a).

The Department of Livestock Development, Thailand has established a conservation program for native cattle genetic resources, including White Lamphun (WL) and Ko Lan (KL) cattle. These two cattle breeds have evolved over generations to adapt to the local conditions of climate and diseases. They may harbor resistance genes which will be used for selection of disease-resistance traits. To date, however, little is known about the genetic variation in the *Slc11a1* gene for native Thai cattle. The present study cloned and characterized the complete coding region (cDNAs) and the 3'UTR of the *Slc11a1* gene in WL and KL cattle and compared the positions of genetic variation found in Thai cattle to the published GenBank sequence of other Zebu and taurine breeds.

MATERIALS AND METHODS

Blood specimen collection

Two breeds of native Thai cattle, White Lamphun breed (n = 5) and Ko Lan breed (n = 5) were selected from the Livestock Research and Testing Station in Phrae and Ratchaburi provinces, respectively. The animals were healthy and had no history of closed-relationship. Blood samples of each animal were collected from the jugular vein into 4.5-mL Vacutainer blood collection tubes with 3.2% buffered sodium citrate solution (BD; Franklin Lakes, NJ, USA), and spun at 1,500×*g* for 10 min. The buffy coat (white blood cells, WBCs) layer was transferred into a new 1.5 mL tube containing hypotonic lysis buffer which destroyed any remaining red blood cells. The WBCs were separated by centrifugation, washed several times with phosphate-buffered saline solution and submerged in RNAlater (Ambion; Austin, TX, USA) to preserve RNA during transportation and storage at -80 °C.

RNA and DNA extraction

Total RNA was extracted from the WBCs using Trizol (Invitrogen; Carlsbad, CA, USA) following the manufacturer's instructions. After RNA extraction, the remaining organic phase was mixed with 100% ethanol and genomic DNA was precipitated by centrifugation at 2,000×*g* for 5 min at 4 °C. The DNA pellet was washed with 10% ethanol with 0.1 M sodium citrate and resuspended in 8 mM NaOH. The quality and concentration of RNA and DNA were measured at OD_{260} and OD_{280} with a spectrophotometer.

Primer design

Two pairs of gene-specific primers (Table 1) were designed using the bovine Slc11a1 cDNA sequence available in the GenBank database (accession number U12862) in order to amplify two polymerase chain reaction (PCR) overlapping fragments (1,120 bp and 860 bp), corresponding to the open reading frame (ORF) of the *Slc11A1*. An additional pair of primers (3-UTR-F and 3-UTR-R) was used for PCR amplification of 3'UTRs.

Reverse transcription polymerase chain reaction amplification of the *Slc11A1* cDNA

The *Slc11A1* cDNA was obtained by reverse transcription polymerase chain reaction (RT-PCR) with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche; Mannheim, Germany). Briefly, Total RNA (1 μg) and anchored-oligo $(dT)_{18}$ primer (Roche; Mannheim, Germany) was denatured by heating at 65 °C for 10 min and then reverse transcribed with the reactions containing 50 mM Tris-HCl (pH 8.5), 30 mM KCl, 8 mM $MgCl_2$, 10 mM of each dNTP, 5 mM DTT, 20 U RNase inhibitor and 10 U Transcriptor High Fidelity reverse transcriptase (final volume 20 μL). The reaction was performed at 50 °C for 30 min using the GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems; Foster City, CA, USA) with heat inactivation at 85 °C for 5 min.

Amplification of the PCR fragments was performed using a 50 μL reaction containing 1 μL first-strand cDNA template with a final volume of 1× PCR buffer, 0.2 mM dNTPs, 0.2 μM of primers, 1.5 mM $MgCl_2$ and 1U Platinum Taq DNA Polymerase (Invitrogen; Carlsbad, CA, USA). The PCR cycling conditions started with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s and a final extension at 72 °C for 10 min.

Polymerase chain reaction amplification of the 3'UTR of *Slc11A1* gene

The same PCR protocol with a new primer pair (3-UTR-F/3-UTR-R) was employed to amplify the 3' UTR of the *Slc11A1* gene. The PCR cycling conditions were an initial denaturation at 94 °C for 3 min, then 35 cycles with denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s and then a final extension at 72 °C for 7 min.

Table 1 Primers for cattle *SLC11A1* gene.

Primer name	position	Primer sequence (5' > 3')	Product size
NR1F	35–53	F CAGCCACTCGCACAGAGAG	1,120
NA1R	1135–1154	R AGATGTACAGGGCTGGAGGA	
NMF2	965–984	F TTTGTCATGGCTGTCTTTGG	860
NPR2	1805–1824	R CTTGCTGCCTTCACACACAT	
3-UTR-F	1691–1710	F GATCAGGAGAAGGGGAGGAC	290
3-UTR-R	1959–1976	R TTGGCTGCCTTCCAGAAC	

A = Adenine, C = Cytosine, G = Guanine, T = Thymine.

Cloning and analysis of polymerase chain reaction products

PCR products were separated by electrophoresis methods on 1.5% agarose gel. A band of the correct size was cut from the gel, column purified using a QIAquick gel extraction kit (Qiagen; Hilden, Germany) and cloned into a TA cloning vector pCR4®-TOPO® with a TOPO TA Cloning ® kit for Sequencing (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA construct was subsequently transformed into *Escherichia coli* cells (One Shot ® TOP10 competent cells; Invitrogen; Carlsbad, CA, USA) and cultured on an agar lysogeny broth plate. At least 2–3 bacterial colonies were selected for larger scale amplification and purification of plasmids. The size of the insert was verified by restriction enzyme analysis, and the plasmids were subsequently sent to the First BASE laboratories (Selangor, Malaysia) for sequencing in both directions using the ABI 3730 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA) with the ABI BigDye (R) Terminator DNA sequencing chemistry kit version 3.1 (Applied Biosystems; Foster City, CA, USA).

The full-length Slc11A1 cDNA was obtained by manual assembly of overlapped PCR sequences and the deduced amino acid sequence of the protein were generated and analyzed by computer analysis. Transmembrane domain predictions were analyzed using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The percent identities of each predicted amino acid sequence of Thai cattle *Slc11A1* (in the present study) were analyzed and compared with known cattle *Slc11A1* sequences obtained from the NCBI GenBank database using the Needle program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), and multiple protein sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/ index.html). From these alignments, phylogenetic trees were constructed using the neighbor-joining algorithm with 1,000 bootstrap replications and a p-distance model implemented in MEGA version 4.0 (Tamura *et al.,* 2007). GenBank sequences used in comparisons were as follows: Zebu Brahman *Slc11A1*(ABF61464), Chinese Holstein *Slc11A1* (NP777077), Hereford *Slc11A1* (DAA32391), Blanco Orejinegro *Slc11A1* (ABF61463), Angus *Slc11A1* (ABM81484). A *Slc11A1* sequence of water buffalo (ABG46344) was used as an out-group and any gaps were removed from the sequence using the MEGA program.

RESULTS

cDNA sequence of native Thai cattle *Slc11A1*

The full length cDNA sequence of the native Thai cattle *Slc11A1* were completed by manual assembly of two overlapping fragments and then deposited in the GenBank database (accession numbers, KF306234–KF306243). The sequence covers a total length of 1773–1790 nucleotides, containing a similar 39 bps of the 5' non-coding region, variable lengths (range from 90 bp to 104 bp) of the 3'- non-coding region and an open reading frame (ORF) of 1,641 bps (n = 4) or 1,644 bps (n = 6). The short ORF (1,641 bps) encodes a 547-amino acid protein with a predicted molecular mass of 59.4 kDa and an isoelectric point (pI) of 7.02. The long ORF (1,644 bps) encodes a 548-amino acid protein with a predicted molecular mass of 59.6 kDa and predicted pI of 6.66 (Figure 1). The deduced amino acid sequences were predicted to have common features of the *Slc11A1* protein found in the other cattle breeds: 12 hydrophobic transmembrane domains (TM), 1 amino-terminal SH_3-binding domain, 4 protein kinase C phosphorylation sites (S/T-X-R/K), 1 N-linked glycosylation sites (N-X-S/T), and 1 consensus transport motif (between TM8 and TM9).

Comparisons of the sequences among native Thai cattle breeds (WL, n = 5 and KL, n = 5) showed six single nucleotide polymorphic (SNP) sites and a 3-nucleotide (GAG) deletion

polymorphism within the coding region of *Slc11A1* gene (Table 2). Three silent mutations were found at nucleotide position 15 (c.15G>A) and 87 (c.87G>A) in exon 2 and 741 (c.741T>C) in exon 8. Three missense mutations were identified in exon 9 at nucleotide position 815 (c.815C>T) with the corresponding the amino acid substitution of an alanine with a valine at codon 272 (p.V272A); in exon 10 at nucleotide position 961 (c.961A>G) with the corresponding the amino acid substitution of an asparagine with an aspartic acid at codon 321 (p.N321D); and in

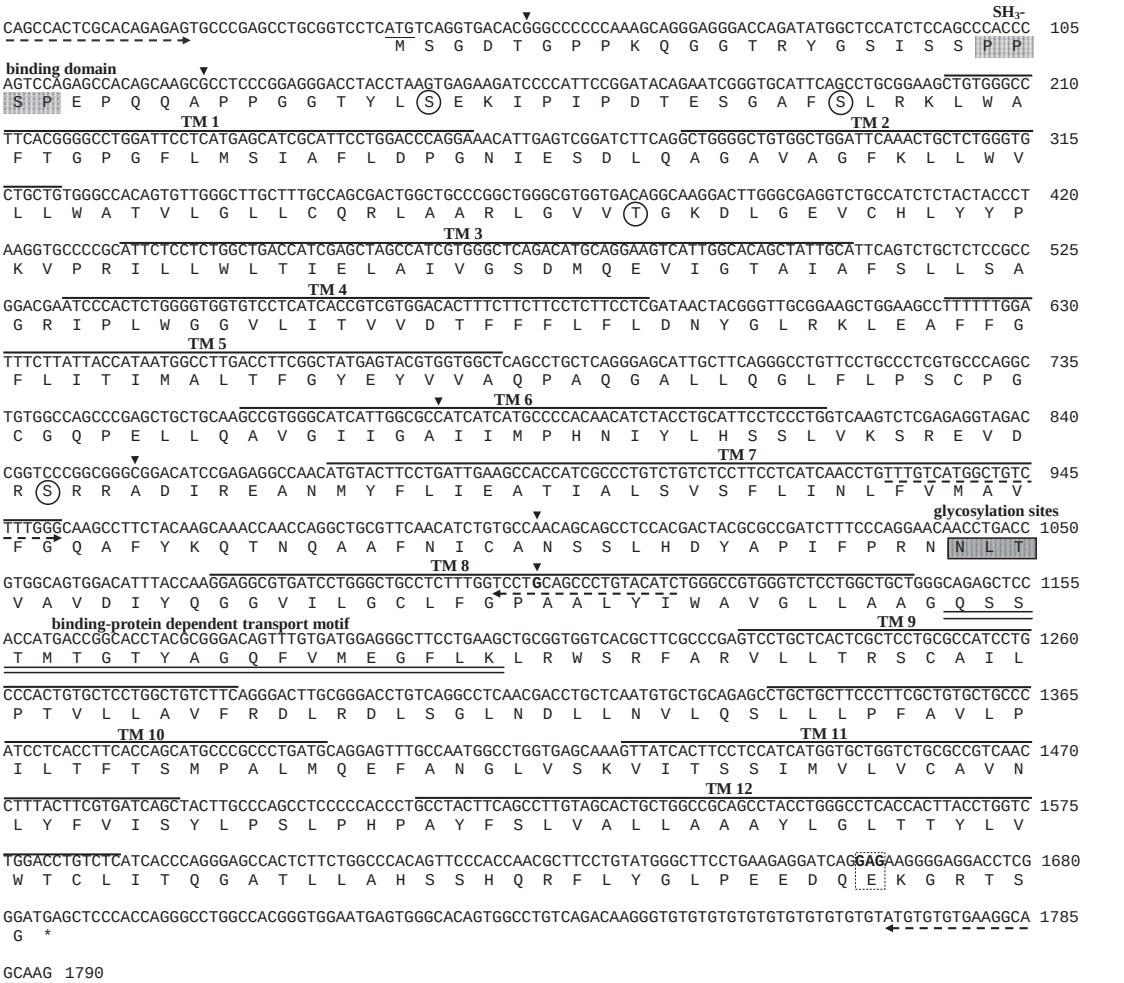

Figure 1 Nucleotide and predicted amino acid sequences of native Thai cattle (White Lamphun) *Slc11A1*. The start codon is underlined and the stop codon is indicated by an asterisk (*). Two sets of primers used to amplify the full-length sequence are represented by dashed arrows underneath the corresponding nucleotide. Lines above the sequence are the predicted hydrophobic transmembrane domains (TM1-12). Arrowheads indicate the positions of single nucleotide polymorphisms (SNPs), and the position of 3-nucleotide (GAG) deletion is boxed with a dashed line. The putative conserved regions are shown as follows: a SH_3-binding domain at amino acid position 21-24 (shaded), four protein kinase C phosphorylation sites (S/T-X-R/K) (circled), a N-linked glycosylation sites (N-X-S/T) (shaded and boxed), and the binding-protein dependent transport motif located between TM8 and TM9 (double underlines).

exon 11 at nucleotide position 1066 (c.1066G>C) with the corresponding the amino acid substitution of an alanine with a proline at codon 356 (p.A356 P). The deletion of three nucleotide at position 1624-1626 (c.1624_1626delGAG) in exon 15 resulted in an in-frame deletion of a single amino acid (glutamic acid) at codon 542 (p.E542del). By comparing the Thai cattle data with available cattle sequences in GenBank, two additional SNPs at nucleotide position 145 (c.145G>A) and 157 (c.157C>A) in exon 3 were identified in the case of the Holstein breed (Table 2). The predicted *Slc11a1* protein of native Thai cattle shared a very high degree of amino acid identity, ranging from 99.5–100% for animals within each breed, and 99.3–100% for animals between the two native breeds (Table 3). Similarly, the Thai cattle *Slc11a1* protein shared 99.3–99.8% and 99.3–100% with the GenBank database known *Slc11a1* protein of Zebu Brahman (*Bos indicus*) and European (*Bos taurus*) cattle, respectively (Table 3).

Table 2 Polymorphisms of the *Slc11A1* cDNA sequences (ORF) in cattle.

Exon number	2	2	3	3	8	9	10	11	15
Nucleotide position	15	87	145	157	741	815	961	1066	1624–1626
White Lamphun 1	G	G	G	C	T	C	A	G	GAG
White Lamphun 2	.	.	.	.	C	.	.	.	-
White Lamphun 3	.	.	.	.	C	.	.	.	GAG
White Lamphun 4	A	.	.	.	C	T*	.	C*	GAG
White Lamphun 5	.	A	.	.	C	.	.	.	-
Ko Lan 1	.	A	.	.	C	.	G*	C*	GAG
Ko Lan 2	.	A	.	.	C	.	G*	.	GAG
Ko Lan 3	.	.	.	.	C	.	G*	.	GAG
Ko Lan 4	.	.	.	.	C	.	.	.	-
Ko Lan 5	.	.	.	.	C	.	.	.	-
Zebu Brahman	.	.	.	.	C	T*	.	.	-
Chinese Holstein	.	A	A*	A	C	.	G*	C*	GAG
Blanco Orejinegro	.	A	.	.	C	T*	G*	C*	GAG

A = Adenine, C = Cytosine, G = Guanine, T = Thymine, * = Missense mutations.

Table 3 Amino acid identity of *Slc11A1* with other known *Slc11A1* proteins.

		White Lamphun (WL)					Kolan (Kl)				
	Length	1	2	3	4	5	1	2	3	4	5
WL 1	548	**100**	99.8	100	99.5	99.8	99.6	99.8	99.8	99.8	99.8
WL 2	547	99.8	**100**	99.8	99.6	100	99.5	99.6	99.6	100	100
WL 3	548	100	99.8	**100**	99.5	99.8	99.6	99.8	99.8	99.8	99.8
WL 4	547	99.5	99.6	99.5	**100**	99.6	99.5	99.3	99.3	99.6	99.6
WL 5	547	99.8	100	99.8	99.6	**100**	99.5	99.6	99.6	100	100
Kl l	548	99.6	99.5	99.6	99.5	99.5	**100**	99.8	99.8	99.5	99.5
Kl 2	548	99.8	99.6	99.8	99.3	99.6	99.8	**100**	100	99.6	99.6
Kl 3	548	99.8	99.6	99.8	99.3	99.6	99.8	100	**100**	99.6	99.6
Kl 4	547	99.8	100	99.8	99.6	100	99.5	99.6	99.6	**100**	100
Kl 5	547	99.8	100	99.8	99.6	100	99.5	99.6	99.6	100	**100**
[a]*Bos indicus*	547	99.6	99.8	99.6	99.8	99.8	99.3	99.5	99.5	99.8	99.8
[b]*Bos taurus 1*	548	99.5	99.3	99.5	99.3	99.3	99.8	99.6	99.6	99.3	99.3
[c]*Bos taurus 2*	548	99.6	99.5	99.6	99.5	99.5	100	99.8	99.8	99.5	99.5
[d]*Bos taurus 3*	548	99.5	99.3	99.5	99.6	99.3	99.8	99.6	99.6	99.3	99.3
[e]*Bos taurus 4*	548	100	99.8	100	99.5	99.8	99.6	99.8	99.8	99.8	99.8

[a]Zebu Brahman ABF61464
[b]Chinese Holstein NP777077
[c]Hereford DAA32391
[d]Blanco Orejinegro ABF61463
[e]Angus ABM81484

Phylogenetic analysis of native Thai cattle *Slc11A1*

To analyze the relationship among the cattle *Slc11A1*, the predicted amino acid sequences of two Thai native cattle breeds (WL and KL) were compared with Brahman and European cattle breeds (Hereford, Blanco Orejinegro, Holstein and Angus). Phylogenetic analysis showed that some WL and some KL *Slc11A1*s were closer in sequence to the Zebu Brahman (*Bos indicus*) *Slc11A1* than to European (*Bos taurus*) *Slc11A1*s and vice versa (Figure 2).

However, the low bootstrap support (less than 50%) indicated that this tree is unlikely to represent the true phylogenetic relationship of the sequences. On the other hand, the neighbor-joining tree indicates that *Slc11A1*sequences were shared across the cattle breeds.

Polymorphic $(GT)_n$ microsatellite repeat within 3'UTR region of *Slc11a1* gene

Three guanine-thymine repeat (GT_n) regions named "GT blocks" at nucleotide positions 1781–1804 (block 1), 1874–1883 (block 2) and 1908–1933 (block 3) in the 3'-UTR of *Slc11A1* DNA sequence were observed in Thai native cattle, similar to previous reports (Feng *et al.*, 1996; Horín, *et al.*, 1999). The GT block1 and GT block3 were polymorphic microsatellites. Due to the T to G substitution at position 1782, three alleles of the GT repeat were identified in GT block 1, designated $^{1782}G + (GT)_{10} = (GT)_{10}$, $^{1782}T + (GT)_{10} = (GT)_{11}$, and $^{1782}T + (GT)_{11} = (GT)_{12}$, followed by the AT dinucleotide that interrupted the GT repeat at position 1805. In the GT block 3, four alleles (GT_{13}, GT_{14}, GT_{15} and GT_{16}) were identified (Table 4) and 16 GT repeats were the most common alleles.

DISCUSSION

The bovine *Slc11A1* gene has been identified and mapped to chromosome BTA 2 within syntenic loci conserved on HSA 2q and MMU 1 (Feng *et al.*, 1996). Two full-length cDNA sequences encoding 547 amino acid proteins (Zebu breeds) or 548 amino acid proteins (European breeds) have been determined. Polymorphisms within bovine *Slc11A1* and their association with disease resistance have also been reported. However, little is known about the molecular characterization of the *Slc11A1* in native Thai cattle. In the present study, RT-PCR was used to clone a full-length cDNAs encoding *Slc11A1*

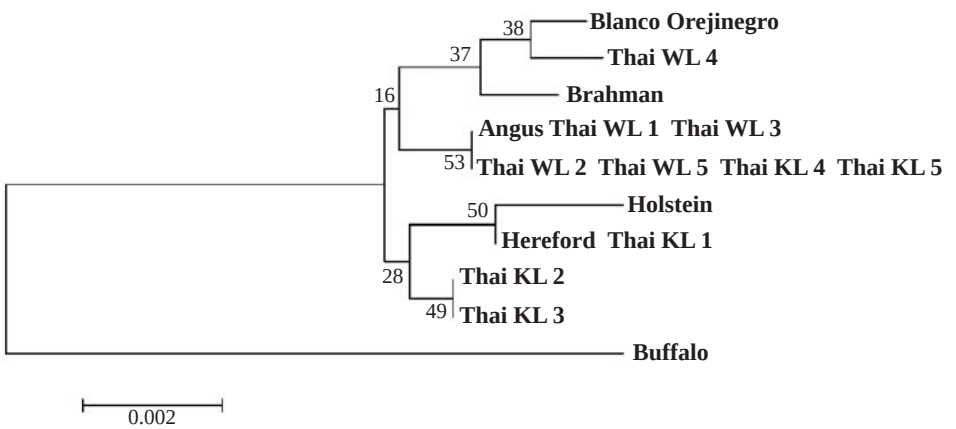

Figure 2 Phylogenetic tree constructed based on the amino acid sequences of bovine *Slc11A1*. The tree was constructed using the neighbor-joining method using the MEGA 5.0 software. Numbers indicate bootstrap percentage (1,000 replicates), and the scale bar represents the number of amino acid substitutions per site. WL = White Lamphun, KL = Ko Lan.

from two native cattle breeds of Thailand (White Lamphun, WL and Ko Lan, KL) and variations in the sequence were determined from five WL cattle and five KL cattle by direct sequencing. Two distinct full length cDNAs encoding 547 or 548 *Slc11A1* proteins were isolated from the two native cattle breeds of Thailand. The sequence is a typical member of the solute carrier family 11 (proton-coupled divalent metal ion transporters) family, containing 12 predicted transmembrane (TM) domains and a consensus transport motif located between TM8 and TM9, conserved between cattle.

It is supposed that native Thai cattle are descendants of the Zebu (*Bos indicus*) breed. In the present study, therefore, it was expected that Thai native cattle, as a closely related species, should have a higher percentage identity for the *Slc11A1* sequence to the Zebu than the European breed. This assumption, however, was not supported. Comparison of the percentage amino acid identities among the *Slc11A1* sequences of native Thai cattle with those foreign cattle breeds showed that WL cattle shared 99.5–100% identity with each other and 99.3–100 % identity with KL and foreign cattle (Table 3). Similarly, the identity levels for amino acid (Table 3) among KL cattle ranged between 99.5 and 100% and between KL and WL or foreign cattle ranged between 99.3 and 100%. Two WL cattle shared 100% amino acid sequence identity with Angus cattle and one KL animal shared 100% amino acid sequence identity similar to the Hereford breed (Table 3). There was only 99.3–99.8% amino acid identity between native Thai cattle and Zebu *Slc11A1* (Table 3). One explanation could be the genetic improvement of native Thai cattle involved the crossbreeding of Zebu and taurine cattle. Analysis of genetic diversity of Asian domestic cattle also showed that native cattle in Thailand were grouped into two subclades, the *Bos taurus* clade and the *Bos indicus* clade (Kikkawa *et al.*, 2003). In fact, nearly all individual Zebu cattle in Southeast Asia contained genetic influence from taurine cattle (Kikkawa *et al.*, 1995).

Further investigation was undertaken of genetic variations in the *Slc11A1* coding region and found three silent SNPs (c.15G>A and c.87G>A in exon 2 and c.741T>C in exon 8) and three missense SNPs (c.815C>T in exon 9, c.961A>G in exon 10 and c.1066G>C in exon 11). All three nonsynonymous SNPs described in this report have previously been identified in different cattle breeds by Martínez *et al*. (2008a). The functional significance of the polymorphism remains unclear. However, Martínez *et al*. (2010) reported that an SNP (p.N321D) in exon 10 of the bovine *Slc11A1* gene had a significant association with the capacity

Table 4 Summary of alleles of Thai cattle *Slc11A1* 3′ untranslated region.

Breed	Position	Sequence (5'→ 3')	Allele block 1	Allele block 3	Reference
		1782 ▼ **block 1**			
Angus	1771–1820	TCAGACAAGGG***T***GTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGAAGGCAG	$(GT)_{12}$	$(GT)_{13}$	U12862
	1821–1870	CAAGACAGACAGGGAGTTCTGGAAGCTGGCCAACGTGAGTTCCAGAGGGA			
		block 2 **block 3**			
	1871–1920	CCTGTGTGTGTGTGACACACTGGCCTGCCAGACAAGGGTGTGTGTGTGTG			
	1921–1970	TGTGTGTGTGTGTGCATGCACAGCAAGACGGAGAGGGAGTTCTGGAAGGC			
White Lamphun (n = 3) and Ko Lan (n = 3)			$(GT)_{11}$	$(GT)_{16}$	This study
White Lamphun (n = 2) and Ko Lan (n = 2)			$(GT)_{10}$	$(GT)_{16}$	
White Lamphun* (n = 1)			$(GT)_{10}$	$(GT)_{15}$	
White Lamphun* (n = 1) and Ko Lan* (n = 1)			$(GT)_{12}$	$(GT)_{14}$	
White Lamphun* (n = 1)			$(GT)_{11}$	$(GT)_{13}$	

* = Individual with two different alleles.

of macrophages to control bacterial growth *in vitro*, but no association with the *in vivo* antibody response stimulated by a challenge. In addition, a mutation at codon 356 (p.A356 P) in exon 11 of the *Slc11A1* gene showed significant association with susceptibility of Holstein-Friesians to Mycobacterium avium ssp. paratuberculosis (MAP) infection (Ruiz-Larrañaga *et al*., 2010).

According to previous publications, the substitution of glycine with aspartic acid at codon 169 (p.G169D) within the TM4 of murine *Slc11A1* has been shown to be responsible for susceptibility to several pathogens (Vidal *et al.* 1995; Govoni *et al.* 1996). However, the p.G169D mutation has not been detected in any other bovine species, including native Thai cattle. Of particular interest in the current study is the three nucleotide (GAG) deletion at codon 542, resulting in a glutamate deletion (p.E542del) in exon 15. This mutation results in a 547-amino-acid protein that contains a cytoplasmic tail that is one residue shorter than that produced by the normal gene. The best available knowledge indicates *Slc11A1* proteins reported in cattle (Feng *et al.*, 1996), sheep and deer (Matthews and Crawford, 1998) have a length of 548 amino acids. The C-terminal deletion of glutamate at codon 542 was reported only in the cattle and rarely observed in most taurine breeds (Martínez *et al*., 2008a). In contrast, the Zebu population has a frequency of greater than 45% of the exon 15 deletion (Martínez *et al*., 2008a). The present study showed both 547- and 548-amino-acid *Slc11A1* protein in the population of Thai native cattle breeds. Two (2/5) WL cattle and two (2/5) KL cattle carry at least one copy of the *Slc11A1* gene encoding a 547-amino-acid protein. Since the p.E542del genotype is rarely found in European cattle breeds, the deletion allele at this position may attract little attention from most researchers and additional studies would be required to identify an association between a 542-deletion allele and disease susceptibility or resistance in Thai cattle population.

To date, only microsatellite $(GT)_n$ polymorphism at 3'UTR of the *Slc11A1* gene has been reported to be linked to natural resistance against brucellosis in cattle (Adams and Templeton, 1998; Barthel *et al.*, 2001). In the GT block 1, two microsatellite $(GT)_n$ alleles , designated $^{1782}G + (GT)_{10} = (GT)_{10}$ and $^{1782}T + (GT)_{11} = (GT)_{12}$ have been reported (Horín *et al.*, 1999; Martinez *et al.*, 2008a). Previous studies demonstrated that the $(GT)_{12}$ allele shows a high frequency (100%) in Holstein cattle (Paixão *et al.*, 2006) and was common in both *Bos taurus* (Blanco Orejinegro) and *Bos indicus* (Zebu) breeds. In addition, the $(GT)_{12}$ allele was associated with natural resistance against brucellosis (Martinez *et al.*, 2008b). In contrast, the $(GT)_{10}$ allele was only found in Zebu cattle (Martinez *et al.*, 2008b). Recently, a novel allele $^{1782}T+(GT)_{12} = (GT)_{13}$ was identified in some cattle populations in Argentina (Hasenauer *et al.*, 2013). In the present study, both the $(GT)_{10}$ and $(GT)_{12}$ alleles were identified in Thai native cattle. In addition, a new allele, designated $^{1782}T + (GT)_{10} = (GT)_{11}$ was identified and represented the most common allele in both White Lamphun (4/5) and Ko Lan (3/5) cattle, respectively (Table 3). However, the $^{1782}T+(GT)_{12}$ allele was not observed in this study.

In the GT block 3, variation in the number of $(GT)_n$ repeats ranged from $(GT)_{13}$ to $(GT)_{16}$ (Barthel *et al.*, 2000, 2001). A marked difference in the $(GT)_{13}/(GT)_{13}$ allele frequency between the Zebu (31%) and Holstein cattle (100%) was found (Paixão *et al.*, 2006) and it was reported that the cattle with the homozygous $(GT)_{13}/(GT)_{13}$ allele were associated with resistance, while other alleles—for example, heterozygous $(GT)_{13}/(GT)_{14\text{-}16}$ or homozygous $(GT)_{14}/(GT)_{14}$ —were associated with susceptibility to brucellosis. In addition, 36% (4 of 11) of the susceptible cattle had a heterozygous $(GT)_{16}$ genotype, while none of the resistant cattle had this allele (Adams and Templeton, 1998). *In vitro* assay using bovine *Slc11A1* transgene into murine RAW264.7 macrophage cells also demonstrated that the level of *Slc11A1* expression obtained from the

susceptible-associated $(GT)_{16}$ allele (*NRAMP1*-S16) cells was three-to-six-fold lower than that of the resistance-associated $(GT)_{13}$ allele (*NRAMP1*-R) cells (Barthel *et al.,* 2001). The $(GT)_{16}$ allele was rarely found in several cattle breeds, both Holstein and Zebu—for example, Gir, GuZera, Nelore (Paixão *et al.*, 2006). In the present study, the $(GT)_{13}$ $(GT)_{14}$ $(GT)_{15}$ and $(GT)_{16}$ microsatellites were identified in the GT block 3 of the Thai cattle *Slc11A1* gene (Table 3). Surprisingly, all 10 cattle observed had $(GT)_{16}$ genotypes. A similar observation was recently published by Hasenauer *et al*. (2013) that $(GT)_{16}$ was the second most frequent haplotype in *B. indicus* breeds. Although the Thai cattle might have been expected to have a very high frequency of the susceptible $(GT)_{16}$ genotype, no data supported the notion that this breed is indeed more susceptible than any of the other breeds in the previous reports. In fact, how the difference in the microsatellite length is implicated in infectious disease resistance/susceptibility remains controversial, since in addition, no association was found between the $(GT)_n$ microsatellite polymorphisms and resistance to brucellosis in some other studies (Kumar *et al.*, 2005; Paixão *et al.*, 2007). In addition, most previous studies that reported the association of *Slc11A1* 3'UTR polymorphism with susceptibility to brucellosis were basing the conclusion from their evaluation of either the GT block 1 or GT block 3, but not from including both the GT repeat regions in the analysis. Moreover, several lines of evidence in humans showed that allelic variants in the 5'-promoter $(GT)_n$ repeat of the gene were also associated with differential *Slc11A1* gene expression (Zaahl *et al.,* 2004) as well as susceptibility both to the infectious agents and to autoimmune diseases (Ouchi *et al.,* 2003; Awomoyi., 2007). Those findings underscore the high *diversity* and complexity in the coding and non-coding regions of the *Slc11a1* gene and suggest that it might be difficult to resolve the relationship between disease resistance and genetic polymorphisms using a single location of the $(GT)_n$ repeat.

CONCLUSIONS

In the present study, the cDNAs and 3'UTR of the *Slc11A1* gene in two Thai (WL and KL) breeds of cattle were characterized. Screening of *Slc11A1* polymorphisms revealed that high allelic diversity of the gene remains in the Thai cattle population. Although only a few animals were used and the results require confirmation from more extensive populations, the findings provide initial information of the *Slc11A1* polymorphisms that allow further investigation of the link between *Slc11A1* genotypes and resistance to any disease in Thai cattle populations.

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